

ACCURATE RECONSTRUCTION OF NEURONAL MORPHOLOGY

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TABLE OF CONTENTS

6.1. Introduction

6.2. Overview of Techniques

6.3. Techniques to reconstruct neurons for simulation

6.3.1. Filling and staining neurons with biocytin in brain slices

6.3.2. Filling and staining neurons with biocytin *in vivo*

6.3.3. Other methods to obtain neuron morphologies

6.3.4. Cross-validation of shrinkage artifacts and dendritic diameter

6.3.5. Using the light microscope to trace stained neurons

6.3.6. Statistical description of neuronal reconstructions

6.3.7. Conversion of morphological description to computer simulations

6.4. List of Suppliers

6.5. Acknowledgements

6.6. References

6.1. INTRODUCTION

To achieve realistic simulations of biological neurons anatomically correct morphological reconstructions of the desired cell type need to be obtained first. If significant errors are made in the reconstruction, the resulting passive and active properties of the simulated neuron could substantially deviate from the real cell. For example, if the diameter of a thin dendrite was traced at 0.5 μm but the actual diameter was 0.8 μm , an error of 60% for the surface area and of 156% for the cross-sectional area would result. Of course, the surface area is proportional to the cell capacitance and total membrane conductance, and the cross-sectional area to the axial conductance, which are important parameters describing the passive structure of neural models (chapter 8). Errors of similar magnitude easily result from omitting the surface area of dendritic spines. Other major sources of error in reconstructions include the degradation of neurons during recording, shrinkage artifacts during histological processing, the omission of faintly stained distal processes from the reconstruction, and just the limitations in the resolution of light microscopy if this technique is used without further validation. This chapter is aimed at providing a step by step description of the techniques that can be used to reduce such problems and to obtain optimal morphological reconstructions for modeling.

6.2. OVERVIEW OF TECHNIQUES

The process of obtaining a reconstructed neuron for modeling typically begins with the injection of a dye during intracellular recording. Alternatives are juxtapositional staining during extracellular recordings *in vivo*, or anatomical stains that do not require recording, such as the Golgi method. Staining during intracellular recording is preferable because the obtained membrane potential traces are very useful as templates for tuning the model later on (see

chapters 8-9). The stained neuron is most commonly visualized with a histological procedure following fixation of the tissue. Fluorescent dyes, however, can be used to visualize neurons before fixation or histological processing take place. Although fluorescent stains are generally not ideal to obtain a detailed reconstruction of the neuron, they can be useful to control for shrinkage artifacts occurring later on. The most widely used technique for visualizing neurons is the injection of biocytin or neurobiotin followed by coupling to avidin-HRP and some variant of a peroxidase reaction with a chromogen to result in a darkly stained neuron. The darkly stained neuron can then be traced under a microscope with a motorized stage using reconstruction software such as Neurolucida (MicroBrightField). Last, the morphology files resulting from this process need to be converted into a format that compartmental simulation software can read.

6.3. TECHNIQUES TO RECONSTRUCT NEURONS FOR SIMULATION

6.3.1. FILLING AND STAINING NEURONS WITH BIOCYTIN IN BRAIN SLICES

6.3.1.1. Slice preparation

The slice preparation method and composition of the slice medium have to be optimized differently for different brain structures. Individual methods to optimize slice quality can not be covered within this chapter, and the literature for the respective brain structure should be consulted. To reconstruct neurons with the biocytin method it is useful to begin the slicing procedure by deeply anesthetizing the animal and then perfusing through the heart with ice-cold slice medium. This washes out erythrocytes from brain capillaries, which contain endogenous peroxidase activity and are therefore darkly stained during the histological protocol described below. Although a fast perfusion (< 1 min after breathing stops) is often advantageous for the physiological quality of slices as well, there may be circumstances where a perfusion is not

possible. In these cases, erythrocyte staining that obscures the visibility of stained neurons can be reduced by an additional step during histological processing (see below).

6.3.1.2. Injection of biocytin

When sharp intracellular electrodes are used, the pipette solution should contain 2-4% biocytin (Sigma) by weight in 1M potassium acetate. This is about the limit in the amount of biocytin that can be dissolved in 1M potassium acetate, and slight warming may be required to get the substance fully dissolved. To fill a neuron, biocytin needs to be ejected with current pulses at the end of the recording. Positive current pulses of 200 ms duration and an amplitude of 1 to 4 nA applied for 5-10 minutes at 1-3 Hz are usually suitable for filling [1,2].

With whole cell recording a concentration of 0.5 to 1% biocytin in the pipette is sufficient and ejection with current is not required. Bulk fluid exchange during recording usually results in a good fill within 5 minutes. Extended recording periods (> 45 min) are not recommended when an optimal morphological reconstruction is desired, because the neuron tends to degrade slowly due to dialysis in the whole cell configuration. The soma may swell, and dendrites may attain a beady or puffy look after prolonged recordings. Similar symptoms can also be due to cell degradation close to the surface of the slice and to progressive slice degradation with time after slicing. The look of dendrites with such poor quality is displayed in Figure 1. For optimal histological reconstructions, recordings of moderate duration early in the experiment and of cells deep in the slice are likely to give the best results. A recording of 15 minutes is usually fully sufficient to give a complete dendritic fill and to stain local axon collaterals if present.

Neurobiotin (Vector Labs) can be used instead of biocytin, and is reputed to have some advantageous properties such as being more soluble and to iontophorese more easily. Background staining that sometimes occurs due to ejection of dye during the approach of a cell with positive pressure (whole cell recording) may also be reduced with Neurobiotin. The

possible disadvantages of biocytin are rarely significant in the experience of the author, however, and the higher expense of purchasing Neurobiotin may not be justified. In addition, a report that Neurobiotin may alter electrophysiological properties of neurons has been published [3].

6.3.1.3. Fixation of slices

When only a light-microscopic reconstruction is desired, the slice can be fixed in 4% paraformaldehyde or in a 10% formaldehyde solution. For convenience, prepared stabilized and buffered fixatives can be purchased, for example from Sigma (HT50-1). When a verification of dendritic diameters with electron microscopy is desired, a suitable EM fixative such as 2.5% paraformaldehyde, 1.25% glutaraldehyde and 15% picric acid in buffer needs to be used [4]. Other authors use less picric acid and more aldehyde fixative [5,6]. For good EM quality it is also essential to avoid prolonged incubation of slices before fixation or extended periods of dialysis during whole cell recording. To obtain good EM ultrastructure from cells recorded in the slice remains a challenge in the best of cases.

6.3.1.4. Histological Processing of Slices

Slices may be left in the fixative for a few hours up to several weeks. Since the visualization of biocytin depends on binding with avidin inside the filled cell, access of the avidin to the cell needs to be ensured. One method is to resection the slice before histological processing [2,6]. A vibratome is suitable to resection slices that have been embedded in agar. To do so, it is important to put the slice flat on a glass slide before embedding to be able to obtain good sections from the entire slice. Overall, however, the author does not recommend resectioning slices, because as a result parts of the stained neuron may be lost and the cell likely will extend through several sections. This may cause serious problems when tracing the morphology of the neuron due to differential shrinkage and deformation of individual sections. In addition, keeping

track of many small branches across sections can be challenging. An alternative to resectioning is permeabilizing membranes throughout the entire slice. This can be done by including 0.1% Triton-X-100 (Sigma #T-9284) in the incubation step of histological processing. The full sequence of steps of histological processing to stain neurons using the Vector ABC kit is listed in Box 1. Slices can be treated individually in small vials that need 1-2 ml of solution for each step. The Vector peroxidase substrate (Vector SK-4700) may be replaced by a standard diaminobenzidine reaction with Ni^{++} intensification. The author prefers the ease of use and low toxicity of the Vector product.

6.3.1.5. Mounting and Clearing of thick Slices

A major problem in obtaining good morphological reconstructions of neurons is tissue shrinkage. Some shrinkage is unavoidable during fixation, and correction factors may need to be determined for this reason (see below). Additional severe shrinkage as well as tissue deformation occurs when thick slices are dried on microscopic slides and dehydrated with increasing alcohol concentrations before clearing with xylene (Figs. 2,3). Although this procedure results in very clear slices and thus highly visible neurons, the accompanying tissue deformation and shrinkage is usually unacceptable. Two alternative protocols can be used. The first protocol consists of clearing floating slices in increasing concentrations of glycerol [7,8]. A one hour immersion in 50% glycerol (Sigma) in buffered water followed by one hour immersion in 90 % glycerol is sufficient. Slices are then mounted and coverslipped in 100% glycerol. This method results in reasonable visibility and little additional shrinkage. Stained neurons, however, tend to fade over the next several weeks. The fading can be largely eliminated by using Immu-Mount (Shandon, Inc.) instead of glycerol. Evaporation of the mounting medium over time can remain a problem even when the slides are sealed with nail-polish. The second method makes use of osmium to harden slices before they are dehydrated. While this method uses highly toxic osmium, and care

needs to be taken to obtain flat slices, it can result in stable and clearly visible stained neurons with minimal tissue shrinkage [9]. A commercially available 4% osmium tetroxide solution (Ted Pella) is diluted to 0.02 – 0.2 % in phosphate buffer and slices are incubated for 5 min to 2 hours depending on slice thickness and the amount of desired tissue hardening. The more osmium is used, the darker the tissue will get, which may ultimately obscure stained neurons. After hardening during osmification, slices can be dehydrated with increasing alcohol concentrations, cleared with xylene, and coverslipped.

6.3.2. FILLING AND STAINING NEURONS WITH BIOCYTIN *IN VIVO*

6.3.2.1. In vivo intracellular recordings

Such recordings are typically performed with sharp electrodes, and the same dye-ejection technique as described above for slice recordings with these electrodes can be used. A key to successful intracellular recording *in vivo* is the reduction of brain pulsations, for example by pouring wax over the exposed brain surface after inserting the electrode [10]. The wax is prepared by mixing paraffin (Electron Microscopy Sciences 19280) with light mineral oil (Fisher 0121) in a waterbath until the mixture has a melting point just above body temperature. The wax is kept melted at this temperature in a waterbath during the experiment. A syringe w/o needle can be used to pour the liquid around inserted electrodes, where it quickly hardens.

At the end of the experiment the animal is typically perfused with buffered saline followed by fixative. The brain should be cut with sections as thick as possible (e.g. 100 μm) to prevent the need for tracing neurons across many sections. Subsequent tissue processing is identical to that of brain slices.

In vivo recordings are common in invertebrate preparations. The dye injection and histological techniques described here are generally applicable to invertebrate preparations as

well. Brain sections are usually replaced by whole-mounts of a ganglion etc., and the tissue can be treated following the guidelines above.

6.3.2.2. Juxtapositional staining

Extracellular recordings can be used to fill neurons *in vivo* with the technique of juxtapositional staining [11]. Recordings are obtained with a glass capillary pulled for intracellular recording, but broken to a tip diameter of 1.5 to 3.0 μm . Breaking can be done by mounting the pulled capillary on a micromanipulator and then advancing the tip into a vertical rough glass surface at an angle of approximately 60 degrees. The process is best done under the microscope using a 20x or 40x objective. This electrode can be filled with 0.5 M or 1.0 M NaCl and 3% biocytin. Extracellular recordings need to be obtained in close proximity to the cells, which can be confirmed by a large spike size. Following recording, biocytin is ejected with 5 to 20 nA positive current pulses. The action potential firing of neurons should accelerate during the applied pulses to indicate close proximity of the electrode tip to the neuron. In a not completely understood fashion the recorded neuron takes up some of the ejected dye and nicely stained cells may be obtained [12]. The histological processing is identical to the case of intracellularly injected neurons.

6.3.3. OTHER METHODS TO OBTAIN NEURON MORPHOLOGIES

6.3.3.1. Photoconversion of fluorescent dyes

In some studies neurons may be filled with fluorescent dyes for particular reasons. For example, the dye may be used for imaging or for localizing dendrites during recording. Fluorescent dyes can subsequently be photoconverted to form a dark reaction product using DAB [13]. This technique is unlikely to achieve the quality of direct biocytin fills, however. In most instances it

seems more advisable to inject biocytin along with the fluorescent dye for later use of the ABC protocol to obtain a dark stain (see above). In some cases injected biocytin may be conjugated to a fluorescent label (e.g. Cy3-conjugated avidin) to allow confocal imaging. If subsequently processed with the ABC protocol, a dark stain can still be obtained [14].

6.3.3.2. Golgi method

Clearly one of the most successful anatomical techniques ever invented, the Golgi method allows the intense full staining of individual neurons in fixed tissue. Most commonly, the rapid Golgi technique that was already known to Cajal is used. Tissue is exposed first to potassium dichromate and then to silver nitrate. For a recent reference to a detailed protocol of the rapid Golgi procedure see [15]. A black deposit fills a small number of neurons fully. Why particular neurons are filled while most others are not remains unknown even after 100 years of use of this technique! A large disadvantage of the Golgi method for neuronal modeling is that physiological data for the stained neurons are not available. Using this technique for modeling is thus not generally recommended. It may be the best technique in special cases, however, like generating models of neurons in human tissue. In addition, a larger sample of cells than could be obtained with intracellular recording might be desirable for the study of morphological variability.

6.3.3.3. Filling individual neurons in fixed tissue

Individual neurons in already fixed tissue can still be filled with dyes. Both fluorescent dyes such as Lucifer Yellow [16], and dyes for a later peroxidase reaction like HRP or biocytin [17] can be used. To monitor the success of injection, the presence of a fluorescent dye is desirable. A conjugate of biocytin and tetramethylrhodamine (Molecular Probes, Inc) appears particularly useful [18]. In distinction to the Golgi technique, one can aim for particular types of neurons with this technique, or stain the detailed morphology of neurons that have previously taken up a

retrograde tracer. As with the Golgi technique, however, electrophysiological data are missing, which makes the obtained morphologies less than ideal for compartmental modeling.

6.3.4. CROSS-VALIDATION OF SHRINKAGE ARTIFACTS AND DENDRITIC DIAMETER

6.3.4.1. Rationale

From the time the brain is removed until coverslipping, neurons may undergo significant changes in morphological structure (Fig. 2). Even just the event of slicing a brain and subsequent incubation may change the morphology significantly by triggering the growth of a sizable proportion of dendritic spines within hours [19]. Thus, it may be difficult to obtain cell morphologies that fully preserve the *in vivo* situation from slice tissue. A comparison with cells of the same type obtained from perfused brains can help judging the presence of slice artifacts such as swelling or spine growth. Both in slices and in perfused brains, neuron morphologies will get distorted to a varying degree during fixation and subsequent histological processing. Before relying on the obtained morphology, some cross-validation of this process is advisable. Of course, the need for such a validation depends on the accuracy that the modeler desires to achieve with morphological reconstructions.

6.3.4.2. Assessing fixation shrinkage

Fixation shrinkage of an entire slice can be assessed by measuring slice size and thickness before and after fixation. Shrinkage factors are estimated from these measurements and applied to the obtained neural reconstructions. These methods assume, however, that shrinkage was uniform throughout the slice and that individual cells shrink at the same rate as the entire slice. This assumption may not hold true in some cases. Shrinkage at the edge of a slice can be different than in the center, leading to a distortion of cells. Also, individual dendrites may curl up rather

than shrink (Fig. 3), and curled dendrites may retain their original length. To assess these possibilities it is desirable to obtain pictures of the filled cell before and after fixation. A fluorescent dye such as lucifer yellow or rhodamine (Molecular Probes) can be injected during recording. These dyes diffuse throughout a neuron within minutes. It should be noted that lucifer yellow in particular is highly phototoxic and neurons can not be exposed to ultraviolet light during electrophysiological recordings. After recording but before slices are fixed, the neuron can be visualized using the correct filter set for the dye used, and pictures can be taken. Water immersion lenses with long working distances are practical to use. For cells with fine dendrites or cells deep in the slice it may be necessary to obtain images with a confocal microscope. After taking a set of pictures of the cell, the tissue is fixed. A second picture of the cell is then taken. Comparing between the pictures before and after fixation, the amount of fixation related shrinkage and distortion of the filled cell can be determined. If precise measurements in the diameter of dendritic processes are desired, measurements should be calibrated by imaging fluorescent beads of known diameter (Molecular Probes, Inc). Following histological processing and coverslipping, a third set of pictures can be taken to assess further shrinkage and deformation.

6.3.4.3. Electron-microscopic methods for detailed analysis of thin processes and dendritic spines

The accuracy of EM is much higher, of course, than that even of confocal techniques. The strength of this technique in the process of making accurate anatomical reconstructions for modeling lies in the ability to make good estimates on the shape and diameter of fine dendrites and spines. Spines can not be traced during LM reconstructions and are often added during the modeling process either just as additional membrane surface or as stereotypical appendages of two compartments each [22]. EM measurements of spines allow for improved accuracy of the

assumed spine characteristics. Since the surface area of spines can contribute more than 50% of the total membrane area of a neuron like in the case of the cerebellar Purkinje cell, a good sample of EM spine measurements may aid greatly in the modeling process. Neurons can be carried to the EM level even after preceding LM analysis [4,5]. Thus, neurons can be traced using LM reconstruction software (see below) and then particular dendritic areas can be singled out for EM analysis. To allow for the use of EM, care has to be taken to preserve tissue ultrastructure as well as possible. The fixative to be used (see above) should be made fresh. Triton needs to be avoided during incubation with avidin. To make sections more permeable, a quick freezing protocol can be used instead. The tissue is cryoprotected (e.g. with 25% sucrose, 10% glycerol in 0.05 M phosphate buffer at pH 7.4) and then rapidly frozen to -80°C and thawed again after 20 min. Slices can be mounted in glycerol for reconstruction under LM, and then washed into phosphate buffer for EM processing. To identify the same dendritic sections with EM that were measured before with LM, a careful alignment strategy is required. Landmarks such as blood vessels or dendritic junctions can be used, and additional marks can be placed by making razor nicks or pinholes in the epoxy-embedded block. The location of these marks can be identified under LM and later used to verify EM positioning.

6.3.4.4. High-voltage EM tomography

Three dimensional imaging of small pieces of dendrite and spines can be achieved with EM methods, when the sample is tilted at various angles under the EM microscope [23,24]. Few scientists have direct access to the required equipment, however. The use of this technique may still be possible through the National Center for Microscopy and Imaging Research under Dr. Mark Ellisman in San Diego (<http://www-ncmir.ucsd.edu/us.html>), which does allow access to its superb equipment. Software that will make the use of the Center's instruments possible across the Internet are under development. Beyond pushing the envelop of EM technology, the

obtained high-resolution images of small dendrites and spines may be of particular use to modelers concerned with the intracellular spatial and temporal dynamics of calcium and second messengers or with the dimensions of synaptic contacts (see chapter 4). Modeling detailed intracellular diffusion processes is becoming an active area of research, since these processes have important implication for ion channel and synaptic dynamics (chapter 3).

6.3.5. USING THE LIGHT MICROSCOPE TO TRACE STAINED NEURONS

6.3.5.1. Limitations

Light microscopy (LM) is limited by the optical resolution that can be obtained. The fundamental limit is approximately $0.6 * \lambda / \text{N.A.}$ where λ is the wavelength of the light and N.A. is the numerical aperture of the objective used [25]. For a typical wavelength of 500 nm and a numerical aperture of 1.0 the limit in resolution is thus 0.3 μm . Numerical apertures of up to 1.5 can be achieved with oil-immersion objectives, but the working distances of high N.A. lenses are relatively short, and generally don't allow focusing deep enough to visualize stained cells in thick brain slices. Water immersion lenses with long working distances and a N.A. of close to 1 are sold both by Zeiss and Olympus. A resolution limit of 0.3 μm does not mean that thinner structures are not imaged. Rather, a thin line of 0.1 μm structure will have an apparent diameter of about 0.3 μm due to light diffraction. This effect leads to an apparent increase in diameter of small dendrites that can lead to a significant error in estimates of axial resistance and membrane surface area. When cells with abundant small profiles are reconstructed for the purpose of accurate passive modeling, a calibration of processes with small diameters using EM is therefore recommended. This is particularly relevant when accurate reconstructions of axon collaterals are desired.

Another problem can arise, when processes take a vertical course through the slice, especially underneath or above the soma. Such profiles may be missed altogether, or the reconstruction may suffer in accuracy. If neurons have a preferred orientation in the target tissue, a slice orientation perpendicular to this orientation should be considered. Cerebellar cortical Purkinje cells provide an extreme example of this point, since these flat cells with extensive branching in two dimensions only would appear as a single line in a horizontal or frontal slice and can not be reconstructed at this orientation at all.

6.3.5.2. Standard LM reconstruction technique using Neurolucida

The cell reconstruction system Neurolucida is sold by MicroBrightField, Inc. To our knowledge, this system is the only commercially available option for 3D computerized single cell reconstructions at the present time. The Eutectics system that is known to many investigators has been discontinued. Most major brand microscopes can be fitted with Neurolucida. A motorized xyz stage is required. It is typically acquired together with Neurolucida, since only a limited number of stage types are supported by the software. A total Neurolucida system including this hardware and computer system (w/o microscope) can be expected to cost around US \$26,000. Several labs have also developed their own reconstruction software, and may make it available in certain circumstances. Information about one of these products (TRAKA) developed in Zürich can be found at <http://www.ini.unizh.ch:80/%7Ejca/>. The following description is based on the use of Neurolucida.

To allow tracing thick slices, a microscope objective with a long working distance and a high magnification needs to be installed. When Neurolucida is installed, great care has to be taken that the stage movement and optical properties of the objectives to be used are properly calibrated. Large errors can result otherwise. It should be noted in particular, that the vertical

movement of the microscope stage by some number of μm does not mean that the specimen is scanned vertically by the same number of μm . Due to the different optical diffraction indexes of glass, oil, and water the movement of the stage can be very different from the distance traversed inside the optical sample. Correction factors need to be determined and set in the software to allow for accurate reconstructions along the z-axis.

To accurately trace the microscopic image of a cell it is required that this image is superimposed with the computer reconstruction. This can be done in two ways with Neurolucida. Either the Lucivid system (MicroBrightField) is used to superimpose the computer drawing onto the microscope image inside the microscope optics, or a camera is mounted on the microscope and the cell image is superimposed with the reconstruction on a computer screen. The latter method is preferred by the author, since looking at a screen is less strenuous than looking through a microscope, and an additional magnification of the image that can be provided by the camera system is useful in judging dendritic diameters.

The actual tracing of a single neuron can take between 30 minutes and several days, depending on the complexity of the neuron. To begin a reconstruction, the soma is traced as a 2D contour around its circumference. Dendrites and axons are attached to the soma and consist of chains of tubular segments. Branch points can be inserted in the reconstruction and subbranches are traced consecutively. The diameter of each segment is chosen to match the superimposed microscope image. This match can be somewhat subjective, and a reconstruction of the same cell by several people can show instructive differences (Fig. 4, Table 1). Processes with thin diameters are particularly prone to large difference when reconstructed by different people. To optimize this process, a sample of thin diameter processes should be cross-checked with other means (see above) and the personnel performing the reconstructions should be instructed to use the resulting criteria of choosing optimal process diameters. The choice of

segment boundaries is another subjective factor in performing reconstructions. Obvious reasons for starting a new segment are a change in process diameter, or a bend in the process. Following a process smoothly in the z-axis requires coordinated use of the z-axis control and the tracing device (mouse or digitization tablet). If the z-axis is only moved when the process becomes quite out of focus a stair-case reconstruction of the process in the z-axis may result. Another problem with the z-axis is the occurrence of a small drift over time. Therefore, when the reconstruction returns to a major branch point several minutes or even hours after it has been placed, a mismatch in the z-axis position of the in-focus image with the previous position stored by the computer may have developed. The current in-focus z-axis position recorded by the computer should be checked against the previous one, and the current z-axis position should be adjusted. If this schedule is not adhered to, an otherwise perfectly good looking reconstruction will suddenly show large vertical excursions at branch points when it is later rotated with other software. While editing such mistakes has become possible with version 3 of Neurolucida, it is still a cumbersome process prone to failure. Because problems may develop over the course of the reconstruction, it is advisable to save backup copies of work in progress.

6.3.5.3. Confocal microscopy for Neurolucida reconstructions

A confocal module can be purchased with the Neurolucida software that allows reconstructions as described above out of confocal stacks. Confocal microscopy has so far not been a good tool to reconstruct detailed morphologies of entire neurons, however. This is largely due to the fact that at the high resolution required for tracing fine branches, a full confocal representation of a neuron will literally extend over hundreds if not thousands of confocal stacks (John Miller, personal communication). In addition, photobleaching generally limits the amount of time fluorescent neurons can be visualized. Nevertheless, confocal microscopy can achieve an approximately 1.4 times improvement in resolution compared to traditional light-microscopic

images [26]. This high resolution can aid in the reconstruction of sample areas of the neuron containing thin profiles.

6.3.6. STATISTICAL DESCRIPTION OF NEURONAL RECONSTRUCTIONS

When Neurolucida is used for neuronal reconstructions, the companion software Neuroexplorer (MicroBrightField, Inc.) is available for a statistical description of the traced neuron. The geometry of the neuron can be drawn as a dendrogram, which is a good visual representation of the branching structure of a neuron. Tables of the number, length, surface area, and volume can be constructed for processes of any order, where order signifies the number of branch points between the soma and the respective process. If a sample of neurons has been reconstructed, the distribution of these values can be plotted to examine whether there are distinct anatomical subpopulations of neurons within the sample. A clear bimodal distribution of the mean dendritic length, distance between branch points, or surface area per dendrite likely indicates the presence of two distinct cell types in the recorded sample. Measures of total membrane surface and total dendritic length should be interpreted with care in neurons obtained from brain slices, since truncated or missing dendrites have a large impact on these measures.

6.3.7. CONVERSION OF MORPHOLOGICAL DESCRIPTION TO COMPUTER SIMULATIONS

6.3.7.1. File conversion

The morphology files created by Neurolucida or other reconstruction programs need to be converted to a different file format before they can be used as cell description files for the most popular compartmental simulation packages, Genesis (<http://www.bbb.caltech.edu/GENESIS>) and Neuron (<http://www.nnc.yale.edu>). The morphology file formats of all of these applications

have in common that one line in the file describes the diameter, size, and absolute location of a single cylindrical compartment. Manual editing therefore is feasible to convert between the specific reconstruction and simulation file formats. Except possibly for very small cells manual conversion is not recommended, however. Automated conversion tools can be written in script languages such as Perl. For conversions from Neurolucida v3 file format to compartmental representations used by Neuron or Genesis a free Java application named CVAPP is available from the Southampton Archive of Neuronal Morphology [27] and is also included on the accompanying CD. In addition to providing file conversion routines, this application is a general cell editor and display tool (Fig. 5). These features can for example be used to cut away reconstructed axons, if axonal modeling is not intended. Note, however, that the axon can make an important contribution to the passive properties of small neurons, especially if local branching is abundant. Matching fast time constants of a neural recording to a passive model without axon may yield substantial errors in the resulting choice of passive parameters. The CVAPP conversion routines to Genesis and Neuron simulations are in beta testing at the time this chapter is written. The accompanying CD should be consulted for help on using CVAPP. An example of a minimal cell in Neurolucida asci file format (cell.asc) and in Genesis morphology file format (cell.p) after conversion with CVAPP is given in Box 2. The resulting genesis file demonstrates the use of a hierarchical naming scheme for the branching structure of the cell. If a modeler needs to make a new conversion tool, this structure could be used as a model. Hierarchical naming is useful, for example, to reflect the logical position of each compartment in the dendritic tree of a neuron. In Genesis this naming scheme allows that particular areas of the dendrite or branch orders are quickly identified in the simulation and endowed with particular properties, such as a unique ion channel distribution.

6.3.7.2. Recompartmentalizing the electrotonic structure of the obtained morphology

Neural simulations are dependent on the correct electrotonic representation of a neuron, not the exact 3D shape such as angles at branch points. The segmentation of a neuron as it is drawn during reconstruction under the microscope is very unlikely to be a good electrotonic representation of the cell. Long straight stretches of a dendrite can be drawn as a single segment, while curvy parts may be broken into many very small parts. For optimal simulation performance all compartments should have approximately the same electrotonic length, i.e. $l / (r_m/r_i)^{1/2}$ should be constant, where l is the actual length of a compartment, and r_m and r_i are the specific membrane and axial resistance, respectively. The actual electrotonic length of a compartment should never exceed 0.1 length constants, but much smaller values may be required, especially when the membrane resistance decreases radically due to the opening of active conductances. A value of 0.01 length constants may serve as a good rule of thumb. The conversion utility CVAPP mentioned above contains an algorithm that re compartments a neuron to a specific desired electrotonic length per compartment.

6.3.7.3. Databases of neuronal morphologies

It is highly desirable that scientists interested in compartmental simulations should be able to share reconstructed morphologies, given that it requires a lot of effort and time to obtain neural reconstructions of high quality. In this regard the on-line archive of reconstructed hippocampal neurons in Southampton [27] is a promising start. Other efforts of constructing shared neuron databases are under way in the US, but only few labs participate in donating their data. Interesting approaches by several groups can be visited on line (<http://www.nervana.montana.edu/projects/neurosyst>; <http://ycmi.med.yale.edu/SenseLab>; <http://www.bbb.caltech.edu/hbp>). Several issues likely need to be clarified before such databases become widely popular. One issue is that of quality control, for example by including

an assessment of possible errors with morphologies contained in databases. A crucial issue is that of who should finance installing and maintaining such databases. Last not least, a strategy to reward authors for submitting data to common databases needs to be designed. While these issues will likely require general changes in the process of how science operates, the contribution of each investigator towards such goals may provide important stepping stones. To reach the goal of increasingly complex realistic simulations of brain structures a high degree of cooperativity between investigators is certainly in order.

6.4. LIST OF SUPPLIERS

Electron Microscopy Sciences, 321 Morris Rd., Box 251, Fort Washington, PA 19034, (800) 523

5874 fax: (215) 646 8931, <http://www.emsdiasum.com>

Fisher Scientific, Pittsburgh, PA., USA (800) 766 7000 fax: (800) 926 1166,

<http://www.fishersci.com>

MicroBrightField, Inc., 74 Hegeman, Ave, Colchester, VT 05446, USA, (802) 655-9360 fax:

(802) 655-5245, <http://www.microbrightfield.com>

Molecular Probes, Inc. 4849 Pitchford Ave., Eugene, OR 97402-9165, USA (541) 465-8300,

fax: (541) 344 6504, <http://www.probes.com>

Olympus America, Inc., Two Corporate Center Dr., Melville, N.Y. 11727-3157, USA (631) 844-

5000, <http://www.olympusamerica.com>

Shandon, Inc. 171 Industry Dr., Pittsburgh, PA 15275, USA (800) 547 7429

Sigma-Aldrich, Inc., SIGMA: P.O. Box 14508, St. Louis, MO 63178, USA, (800) 325-3010, fax:

(800) 325-5052, <http://www.sigma-aldrich.com>

Ted Pella, Inc, P.O. Box 492477, Redding, CA 96049-2477, USA, (800) 237-3526 fax: (530)

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Carl Zeiss. USA (800) 233-2343 fax: (914) 681 7446. <http://www.zeiss.com/micro>. Note:
Expect shipment delays of several months.

6.5. ACKNOWLEDGEMENTS

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TEXT BOXES

BOX 1

Histological processing of slices to stain neurons filled with biocytin

- 1) Rinse fixed slice 3 times in 0.01 M potassium phosphate buffered saline (KPBS, pH 7.4)
- 2) ONLY IF ANIMAL WAS NOT PERFUSED: Incubate in 1% H₂O₂ + 10% Methanol + 2% albumin in 0.1 M KPBS for 30 min at room temperature while gently shaking. This step reduces endogenous peroxidase activity. Then rinse 3 times in KPBS as above.
- 3) Incubate slice in 0.1 M KPBS with 2% bovine albumin (Sigma A-2153) and Vector ABC solutions (Vector Pk-4000) overnight in fridge while gently shaking. If slices need to be permeabilized, add 0.1% Triton-X100 (Sigma T—9284). Note that the ABC kit A and B solutions need at least 30 min to form a complex after mixing before they can be used.
- 4) Rinse thoroughly 5 times with KPBS.
- 5) Use Vector substrate kit (SK-4700) or a standard diaminobenzidine (DAB) protocol for the final peroxidase reaction. If DAB is used, prepare a solution of 0.06 % NiCl, 0.05% DAB, and 0.06% H₂O₂ for immediate use. Staining takes 10-15 minutes. It should be discontinued earlier if the non-specific background stain becomes very dark.

BOX 2

Neurolucida and Genesis morphology file formats

A: Neurolucida asci representation of a small cell. Segment endpoints are given in absolute x,y,z coordinates and diameter.

```
; V3 text file written for MicroBrightField products.
(Sections)
```

```
("CellBody"
```

```

(Color DarkRed)
(CellBody)
(  -0.93      3.57      0.00      0.12) ; 1, 1
(   3.14      3.57      0.00      0.12) ; 1, 2
(   4.42      1.04      0.00      0.12) ; 1, 3
(   4.30     -1.38      0.00      0.12) ; 1, 4
(   2.56     -2.42      0.00      0.12) ; 1, 5
(  -0.35     -2.30      0.00      0.12) ; 1, 6
(  -2.09     -1.84      0.00      0.12) ; 1, 7
(  -2.67      0.23      0.00      0.12) ; 1, 8
(  -2.33      2.88      0.00      0.12) ; 1, 9
) ; End of contour

( (Color Yellow) ; [10,1]
(Dendrite)
(   0.93      3.69      0.00      1.40) ; Root
(   0.93      6.22      0.00      1.40) ; R, 1
(   1.28      9.45      0.00      1.40) ; R, 2
(   2.09     12.56      0.00      1.40) ; R, 3
(   2.67     15.90      0.00      1.40) ; R, 4
(
  (  10.00     21.77      0.00      1.16) ; R-1, 1
  (
    (  20.35     22.12      0.00      0.93) ; R-1-1, 1
    Normal
    |
    (  11.39     29.37      0.00      0.70) ; R-1-2, 1
    Normal
  ) ; End of split
  |
  (  -5.93     27.42      0.00      1.05) ; R-2, 1
  Normal
) ; End of split
) ; End of tree

( (Color Blue) ; [10,1]
(Dendrite)
(   4.42     -0.12      0.00      1.63) ; Root
(  22.09     -0.35      0.00      1.63) ; R, 1
(
  (  27.91      9.68      0.00      1.16) ; R-1, 1
  Normal
  |
  (  35.35     -7.83      0.00      0.93) ; R-2, 1
  Normal
) ; End of split
) ; End of tree

```

B: Same cell in genesis morphology file format after conversion with cvapp.

The soma contour has been replaced by a single spherical compartment. The coordinate scheme for dendritic compartments is retained.

```

// genesis
//

```

```
// Cell morphology file for GENESIS.  
// Written by cvapp (http://www.neuro.soton.ac.uk/cells/#software).  
  
*absolute  
*asymmetric  
*cartesian  
  
// End of cvapp-generated header file.  
  
*origin 0.706 0.756 0  
  
*compt /library/soma  
soma none 0.706 0.756 0 6.067  
  
*compt /library/dendrite  
p0[1] soma 1.28 9.45 0 1.4  
p0[2] p0[1] 2.09 12.56 0 1.4  
p0[3] p0[2] 2.67 15.9 0 1.4  
p0b1[0] p0[3] 10 21.77 0 1.16  
p0b1b1[0] p0b1[0] 20.35 22.12 0 0.93  
p0b1b2[0] p0b1[0] 11.39 29.37 0 0.7  
p0b2[0] p0[3] -5.93 27.42 0 1.05  
  
p1[1] soma 22.09 -0.35 0 1.63  
p1b1[0] p1[1] 27.91 9.68 0 1.16  
p1b2[0] p1[1] 35.35 -7.83 0 0.93
```

TABLES

	Rec. A	Rec. B	Rec. C	Rec. D
Length Dendrite 1 (μm)	1160	1226.3	1378.1	1307.1
Surface Area Dendrite 1 (μm) ²	4829.09	5230.72	4963.62	4344.62
Branch Points Dendrite 1	8	8	11	15
Length Dendrite 2 (μm)	540.6	508.4	481.3	650.6
Surface Area Dendrite 2 (μm) ²	1899.23	1931.47	1057.34	2321.12
Branch Points Dendrite 2	4	3	5	6
Length Dendrite 3 (μm)	1107.5	1158.4	1133.1	1268.7
Surface Area Dendrite 3 (μm) ²	3980.27	4204.36	2641.09	4147.13
Branch Points Dendrite 3	7	7	8	9
Length Dendrite 4 (μm)	1249.6	1251.9	1242.7	1385
Surface Area Dendrite 4 (μm) ²	5352.67	5535.01	3723.33	5007.53
Branch Points Dendrite 4	11	9	11	20

Table 1: Cell statistics of 4 reconstructions of the same GP neuron. Pictures of the reconstructions are shown in Fig. 4. The surface area of the cell in particular is quite variable between reconstructions. All people performing these reconstructions had previous experience in the use of Neurolucida. Specific instructions as to how to trace thin processes were not given.

FIGURE CAPTIONS

Figure 1: A. A pair of neurons was recorded simultaneously from globus pallidus (GP) in a slice from a 16 day old rat. Both cells were filled with biocytin during whole cell recording and processed with the Vector ABC method. The morphological quality of one neuron (open arrow) was much better than that of the 2nd one (solid arrow), although both neurons were recorded for the same duration with identical technique. The most likely explanation for the difference in this case is that the more degraded neuron was somewhat more damaged during the slicing procedure and had started to degrade before recording. The electrophysiological quality of this cell was

also poor. B. The dendrite of another GP neuron of poor quality is shown at increased magnification. The arrows point to typical signs of degradation, such as beads and patchy staining.

Figure 2: A: Picture of a cerebellar cortical Purkinje cell recorded from a slice of a 14 day old rat and filled with biocytin. This image was taken after fixation and staining, but while the slice was still in buffer solution. B: Picture of the same cell after drying on a glass slide, dehydration, and coverslipping. It is clear that the shape of the cell has become distorted in a non-uniform way. The upper left dendrite in particular looks stretched.

Figure 3: Picture of a GP dendrite. This cell was lightly air-dried on a glass slide, and then dehydrated using alcohol and xylene before coverslipping. The open arrow points at a straight segment of dendrite coursing in the xy plane. The solid arrow points at a segment of dendrite that is ascending in the z-axis. This segment is curled up due to the large shrinkage of the entire slice in the z-axis. Curled dendrites frequently are common when severe shrinkage of slices occurs, indicating that individual processes do not shrink at the same rate as the slice in which they are embedded.

Figure 4: Four pictures of the same GP neuron reconstructed by different people. Some differences in dendritic diameters used can be discerned. The resulting large differences in cell properties and especially cell surface area are listed in Table 1.

Figure 5: Image of the user interface provided by the Java cell viewer cvapp. A GP neuron is displayed in the cell window. Neurons can be rotated in 3D, and morphology files can be written in different formats.

FIGURES

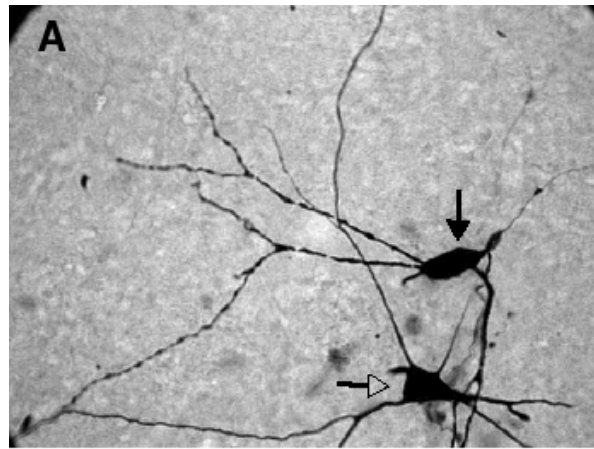


Fig. 1.

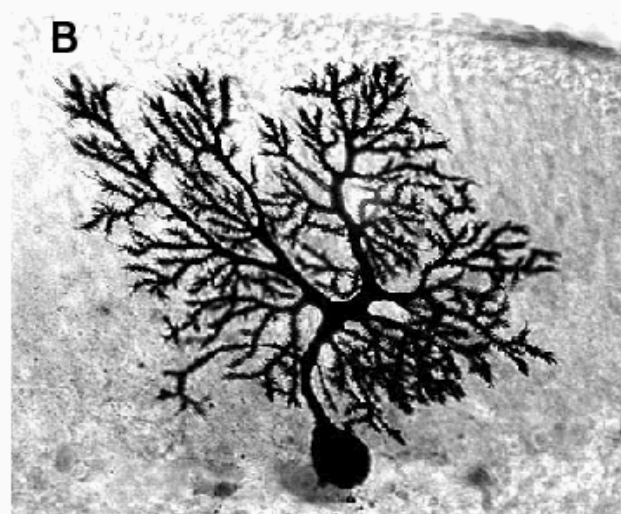
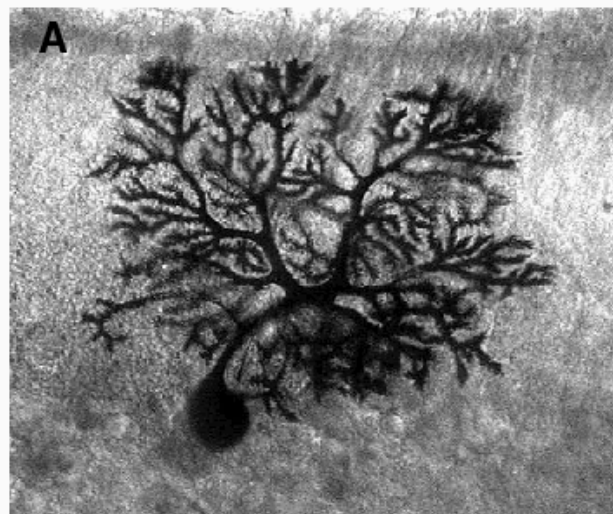
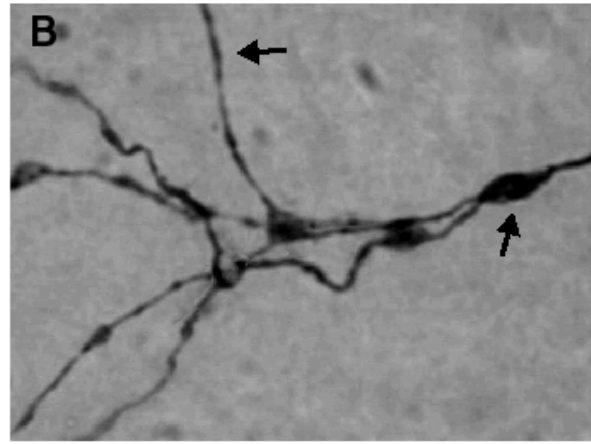
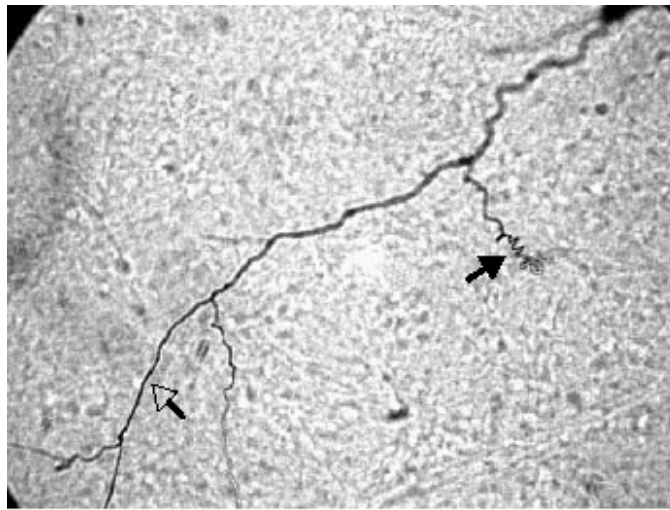


Fig. 2



100 μm
Fig 3.

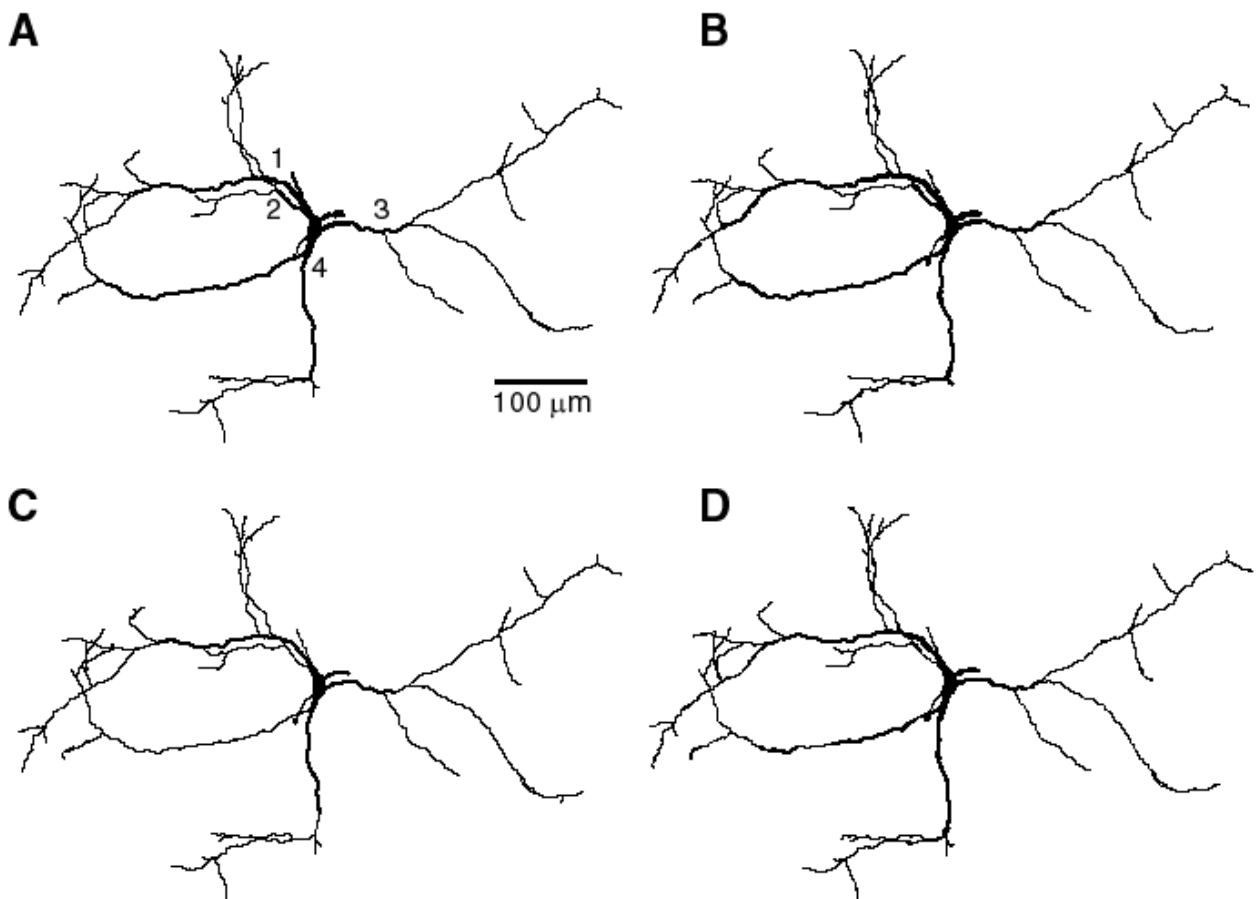


Fig. 4.

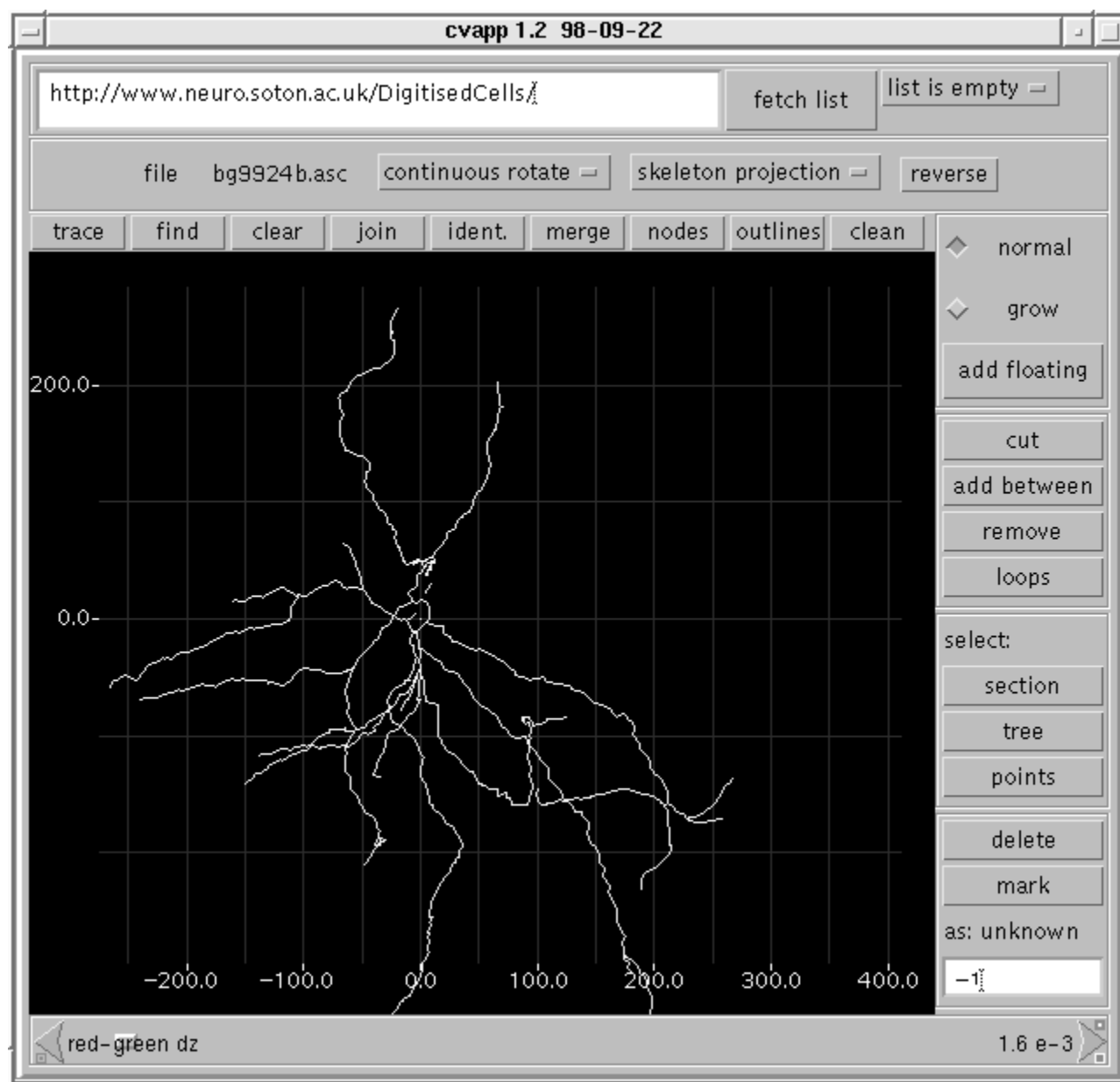


Fig. 5